IMMUNOCHEMICAL STUDIES ON HUMAN PLASMA LIPOPROTEINS*

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Some years ago, a series of human serum lipoproteins, distinguishable by their hydrated densities and lipid-protein ratios were recognized and isolated by ultracentrifugal techniques (1). Certain of these lipoproteins are invariably present in human serum and the concentrations of these and of others can be quantitatively correlated with disease (2). No difference in the lipoprotein distribution can be demonstrated between serum and plasma. The purpose of this investigation was to obtain information about the immunochemical specificity of some of the lipoproteins.

Methods

Preparation of Lipoproteins[1].—All lipoproteins, unless it is otherwise indicated, were isolated from pooled plasma. The plasma was obtained from citrated blood which was rejected by the San Francisco Blood Bank because of blood clots, positive serologic tests for syphilis, or because 3 weeks or more had elapsed since it was drawn. Between 8 and 16 pints of blood were used per pool.

Low density lipoproteins S_f6 and S_f13 were isolated by the method of Lindgren *et al.* (3). The ultracentrifugal homogeneity of similar preparations has been discussed (5). The hydrated density of S_f6 lipoproteins is between 1.03 and 1.04 gm./ml. and that of S_f13 lipoproteins between 1.015 and 1.03 gm./ml. In Fig. 1 ultracentrifuge records of low density lipoprotein preparations are presented.[2] These lipoproteins were used as immunizing antigens

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[1] The lipoprotein nomenclature that will be used throughout is that suggested by Lindgren *et al.* (3) for low density lipoproteins (lipoproteins of hydrated density less than 1.063 gm./ml. and that suggested by deLalla and Gofman (4) for high density lipoproteins (lipoproteins of hydrated densities 1.07 to 1.16 gm./ml.)

[2] Ultracentrifuge analyses were made with a Spinco model E analytical ultracentrifuge at a density of 1.063 gm./ml., 26°C. and a rotor speed of 52,640 r.p.m. (average distance from center of rotation is 6.5 cm.). Only one frame for each preparation, 30 minutes after full rotor speed was attained, is reproduced. No evidence of lipoproteins can be seen in any of the other frames that cannot be seen in the frame shown. The complete records have been presented elsewhere (6). Two preparations were always run simultaneously; some of them were not used in the experiments described but have not been removed from the ultracentrifuge records.

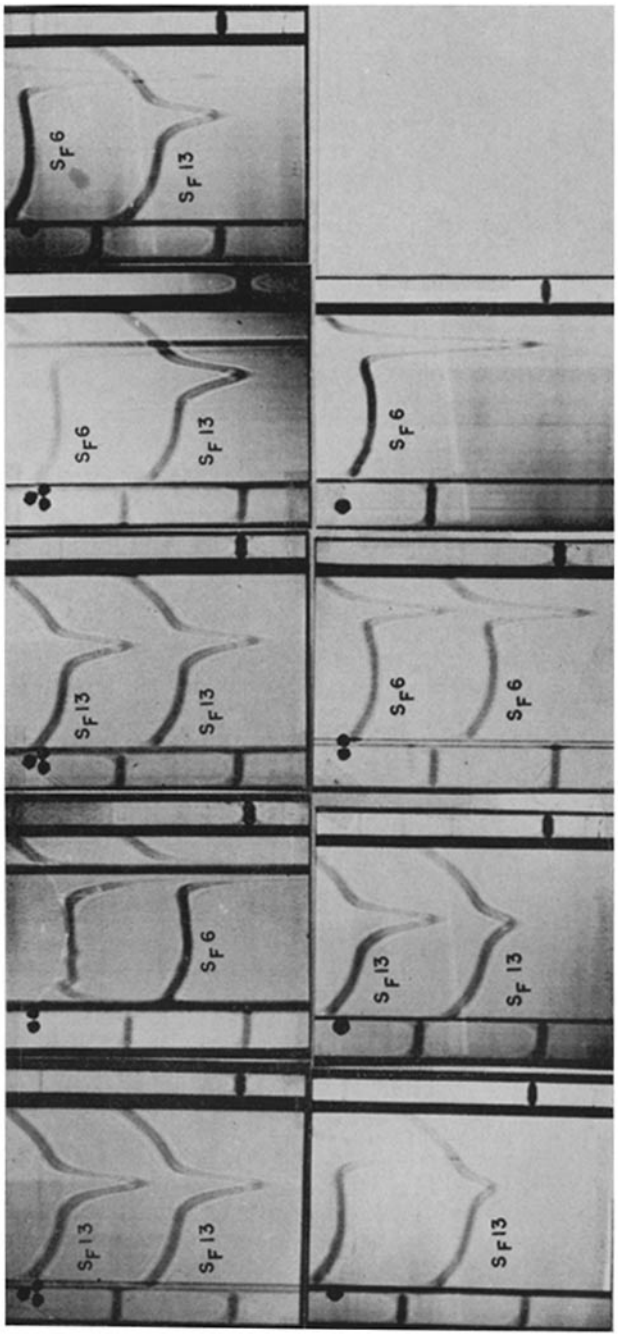

FIG. 1. Ultracentrifuge records of immunizing antigens. Low density lipoproteins $S6_f$, S_f13. For details see text.

(see below). An analysis of the ultracentrifuge records of the various S_f6 and S_f13 preparations (4) indicated the amount of material common to the two types of preparations to be less than 1 per cent of the total lipoprotein concentration. In the S_f6 preparations there were no measurable lipoproteins of flotation rates greater than S_f8 or less than S_f4, except for one preparation that had less than 1 per cent of total lipoprotein between S_f8 and 11. In the S_f13 preparations there were in no case measurable concentrations of lipoproteins of flotation rates above S_f16 or below S_f11.

S_f17+ was the designation given to material collected in the top 0.5 ml. from unaltered plasma that was centrifuged in the preparative Spinco ultracentrifuge, model L, at 30,000 R.P.M., 26°C., for 24 hours in a Spinco No. 30 rotor. The hydrated density of this material was less than 1.015 gm./ml.; it contained principally S_f20 to 40 lipoproteins and small amounts of material up to the chylomicron range.

Two high density lipoprotein fractions were isolated, one of mean hydrated density 1.075 gm./ml., which has been designated HDL-2 (4), and the other, of mean hydrated density 1.145 gm./ml. which has been designated HDL-3. These fractions were isolated by the general method of deLalla and Gofman (4), except for the following modifications; some of the low density lipoproteins were first separated by removing the floated lipoproteins after centrifugation of unaltered plasma for 24 hours at 30,000 R.P.M. in a preparative No. 30 rotor. Most of the remaining low density lipoproteins were then separated at density 1.063 gm./ml. by repeated ultracentrifugation and removal of the floated lipoproteins, followed by dilution of the subnatant in sodium chloride solution of density 1.063 gm./ml. Five recentrifugations at density 1.063 gm./ml. were necessary to remove low density lipoproteins from a preparation containing HDL-2 and HDL-3 lipoproteins. Film analysis of this preparation indicated that the final low density lipoprotein concentration was approximately 0.2 per cent of the total lipoprotein concentration.

Lipoprotein-free plasma protein fractions were prepared from the subnatants of tubes from which the high density lipoproteins were isolated. These subnatants were recentrifuged four times at density 1.21 gm./ml., 30 hours at 40,000 R.P.M. each followed by the removal of the floated lipoproteins and dilution to the original volume with D_2O-salt solution of density 1.21 gm./ml.

Preparation of Antiserums.—Antiserums against low density lipoproteins were prepared by injecting subcutaneously 14 rabbits with S_f6 and 14 rabbits with S_f13. Ultracentrifuge records of all the low density lipoprotein preparations that were used as immunizing antigens are presented in Fig. 1 and are marked S_f6 and S_f13, respectively. The rabbits received 10 injections, (2 or 3 injections on successive days every 2 or 3 weeks) over a period of 2 months, about 10 mg. lipoprotein per injection. 8 days after the last injection the rabbits were bled. 5 months later all rabbits received one more injection of lipoprotein and were again bled after 8 days. All low density lipoprotein fractions were used within 2 or 3 days after isolation since they tended to form a small amount of insoluble precipitate after standing a week or longer in the ice chest at 4°C.

Antiserum against high density lipoproteins was prepared by injecting a lipoprotein preparation containing HDL-2 and HDL-3. High density lipoproteins were found to be more stable to storage than low density lipoproteins since no insoluble precipitate developed during several weeks at 4°C.; one preparation sufficed. Eight rabbits were injected subcutaneously with this preparation thrice weekly for 3 weeks, about 10 mg. lipoprotein per injection. The rabbits were bled 6 days after the last injection.

Fractionation of Antiserums.—Antiserums against low density lipoproteins and pooled normal rabbit serum were fractionated by repeated precipitation with half-saturated ammonium sulfate, followed by separation of the precipitate into the water-soluble pseudoglobulin and the water insoluble euglobulin fractions, as previously reported (7). The pseudo-

globulin fractions of the anti-S_f6 serum pools are referred to as P-6, the euglobulin fractions as E-6. Corresponding designations are given to anti-S_f13 serum fractions, P-13 and E-13.

Salt Concentration of Precipitin Reactions.—Qualitative and quantitative precipitin reactions were carried out at an ionic strength of approximately $\mu = 0.1$. To obtain this salt concentration, antiserum fractions were dialyzed but lipoprotein fractions were diluted with distilled water. They were not dialyzed since changes of lipoproteins upon dialysis have been observed (8).

EXPERIMENTS AND RESULTS

Tests for Cross-Reaction between Lipoproteins and Non-Lipide Containing Proteins

Anti-lipoprotein serums and serum fractions were tested by precipitin tests with the following antigens: human serum albumin, human serum gamma globulin, and the subnatant of a 1.2 density ultracentrifuge run. No precipitate was detected with the first two antigens. It was found that the subnatant did precipitate with anti-lipoprotein serums. After four recentrifugations of the subnatant, as described above, the last traces of lipoproteins were apparently removed and no more precipitate formed with anti-lipoprotein serums.

None of the lipoprotein fractions precipitated with antiserums against human albumin or human gamma globulin.

Comparison of Lipoprotein Fractions from Different Individuals

Serums from freshly drawn blood of seven presumably normal individuals, plasma from 5 individual pints of blood bank blood, and plasma obtained by pooling 8 pints of blood bank blood (*i.e.* 13 samples) were used. We did not consider it necessary to employ the rather cumbersome isolation procedure described on the previous pages. The following simplified procedure was substituted:—

The thirteen test samples were centrifuged in the preparative ultracentrifuge, 9 ml. each, at 30,000 R.P.M. for 24 hours, at 26°C. Four fractions were pipetted from each tube.

(*a*) Fraction 1. The top 0.75 ml.; this fraction contained all the lipoproteins that had floated to the top of the tube. These were of S_f17 and of higher S_f rate, up to and including chylomicrons. The next 3.75 ml. of the clear layer beneath were discarded. This solution contained only very small amounts of lipoproteins, principally of S_f13 to 17. (*b*) Fraction 2. The next 1.0 ml.; this contained most of the lipoproteins of S_f8 to 16. (*c*) Fraction 3. The next 0.5 ml.; this was the material just above the albumin boundary and consisted of lipoproteins of S_f3 to 8 and a small amount of albumin. (*d*) Fraction 4. The next 1.5 ml.; this material contained albumin, globulin, and a large portion of high density lipoproteins HDL-2 and HDL-3. The remaining 1.5 ml. was discarded.

2 ml. of P-6 and 2 ml. of P-13 antiserum fractions from the second bleeding were fractionally absorbed with each of the four fractions from each of the 13 samples until no more precipitate appeared within 48 hours. The absorbed antiserums were then tested with the corresponding fractions of every other samples and with the original sample as control. Unabsorbed antiserum fractions were used as another set of controls.

It was found that once an antiserum preparation was absorbed with a lipoprotein fraction of any one individual, no further precipitation occurred with the same lipoprotein fractions of any other serum or plasma. No difference in

specificity could be demonstrated for any one of the four lipoprotein fractions from twenty individuals.

Comparison of Lipoprotein Fractions Isolated from One Plasma Pool

Qualitative Absorption Analyses.—Antiserum preparations P-6, E-6, P-13, and E-13 from the second bleeding and anti-(HDL-2 and HDL-3) serum were fractionally absorbed with lipoprotein antigens S_f17+, S_f13, S_f6, HDL-2, and HDL-3. When no more precipitate appeared upon further addition of absorbing antigen, the absorbed antiserums and antiserum fractions were divided into five aliquots and each reacted with one of the five antigens. All the anti-low density lipoprotein preparations gave identical results and are presented as a

TABLE I

Reactions of Anti-Lipoprotein Serums with Isolated Lipoproteins before and after Absorption

Antiserums or antiserum fractions	Antigens				
	S_f 17+	S_f13	S_f6	HDL-2	HDL-3
1. P-6, E-6, P-13, E-13					
(*a*) Before absorption	+	+	+	+	−
(*b*) After absorption with: S_f17+	−	−	−	−	−
S_f13	−	−	−	−	−
S_f6	−	−	−	−	−
HDL-2	+	+	+	−	−
HDL-3	+	+	+	±	−
2. Anti-(HDL-2 and 3)					
(*a*) Before absorption	±	±	+	+	+
(*b*) After absorption with: S_f17+	−	−	−	+	+
S_f13	−	−	−	+	+
S_f6	−	−	−	+	+
HDL-2	+	+	+	−	+
HDL-3	+	+	+	+	−

group in Table I, together with the results using the anti-(HDL-2 and HDL-3) serum. The results are discussed below. Precipitation between low density lipoproteins and anti-(HDL-2 and HDL-3) serum absorbed with HDL-2 is assumed to have been due to anti-low density lipoprotein antibodies present in this serum as a result of a small amount of low density lipoprotein contamination (~0.2 per cent) in the immunizing antigen.

Quantitative Precipitin Analyses.—Quantitative precipitin analyses were performed with S_f6 and S_f13 lipoprotein preparations and all antibody fractions. The details of the technique were reported previously (7). Normal rabbit serum and serum fractions were used throughout as controls. Cholesterol analyses were performed on some of the replicate precipitates and supernatants by the method of Colman and McPhee (9). It was uniformly found, in both the homologous and heterologous systems, that all the cholesterol added in the form of lipoprotein antigen was precipitated throughout the region of antibody excess.

Results of quantitative precipitin analyses are given in Tables II to IX and Figs. 2 to 5. Only data obtained with antiserum fractions from the first bleeding and lipoprotein preparations obtained from one plasma pool are given here.

Those from the second bleeding were essentially the same and have been presented in detail elsewhere (6).

Agar Diffusion Analyses.—The agar diffusion technique was performed as described by Oudin (10). The pH of all reaction mixtures was adjusted to 7.1. Tubes were observed for 20

TABLE II

Addition of Increasing Amounts of S_f6 to Anti-S_f6 Rabbit Pseudoglobulin

S_f6 N added	Total N ppt.	1.0 ml. antiserum fraction per tube		
		Antibody N by difference	Ratio antibody N:lipoprotein N in ppt.	Tests on supernatant
mg.	*mg.*	*mg.*		
0.0077	0.0175	0.0099	1.29	Not done
0.0153	0.049	0.0337	2.20	
0.0191	0.0665	0.0474	2.48	
0.0229	0.0805	0.0576	2.51	
0.0306	0.106	0.0754	2.46	
0.0383	0.1225	0.0843	2.20	
0.0478	0.1365	0.0887	1.86	
0.0574	0.1475	0.0901	1.57	
0.0765	0.1435			
0.0956	0.1225			
0.1148	0.0735			
0.1339	0.0525			
0.1530	0.0385			
0.1721	0.000			

TABLE III

Addition of Increasing Amounts of S_f13 to Anti-S_f6 Rabbit Pseudoglobulin

S_f 13 N added	Total N ppt.	1.0 ml. antiserum fraction per tube		
		Antibody N by difference	Ratio antibody N:lipoprotein N in ppt.	Tests on supernatant
mg.	*mg.*	*mg.*		
0.0073	0.0326	0.0254	3.50	Excess antibody
0.0145	0.0466	0.0321	2.21	" "
0.0242	0.0606	0.0364	1.51	" "
0.0483	0.0933	0.045	0.932	No antibody, no antigen
0.0724	0.1167	0.0443	0.613	" " " "
0.0967	0.1352			" " " "
0.121	0.1493			" " " "
0.145	0.1493			Excess antigen
0.169	0.1352			" "
0.1932	0.1167			" "
0.2175	0.0886			" "
0.2415	0.0513			" "

TABLE IV

Addition of Increasing Amounts of S_f6 to Anti-S_f13 Rabbit Pseudoglobulin

S_f6 N added	Total N ppt	1.0 ml. antiserum fraction per tube		
		Antibody N by difference	Ratio antibody N; lipoprotein N in ppt.	Tests on supernatant
mg.	*mg.*	*mg.*		
0.0061	0.0252	0.0191	3.12	Excess antibody
0.0092	0.0364	0.0272	2.97	" "
0.0122	0.0448	0.0326	2.66	" "
0.0153	0.0533	0.038	2.48	No antibody, no antigen
0.0184	0.070	0.0516	2.81	" " " "
0.0245	0.0756	0.0511	2.09	" " " "
0.0306	0.0890	0.0590	1.93	" " " "
0.0383	0.101	0.0628	1.64	" " " "
0.0459	0.1065			Excess antigen
0.0612	0.070			" "
0.0765	0.0448			" "
0.0918	0.0336			" "
0.1071	0.0196			" "
0.1224	0.0084			" "
0.1530	0.0056			" "
0.2448	0.0028			" "

TABLE V

Addition of Increasing Amounts of S_f13 to Anti-S_f13 Rabbit Pseudoglobulin

S_f13 N added	Total N ppt.	1.0 ml. antiserum fraction per tube		
		Antibody N by difference	Ratio antibody N: lipoprotein N in ppt.	Test on supernatant
mg.	*mg.*	*mg.*		
0.0054	0.0175	0.0121	2.22	Excess antibody
0.0190	0.0315	0.0206	1.89	" "
0.0182	0.049	0.0309	1.70	No antibody, no antigen
0.0272	0.0665	0.0393	1.44	" " " "
0.0363	0.084	0.0477	1.31	" " " "
0.0543	0.1018	0.0475	0.87	" " " "
0.0725	0.105			" " " "
0.0906	0.084			Excess antigen
0.1089	0.070			" "
0.127	0.042			" "
0.145	0.028			" "
0.1815	0.0105			" "

TABLE VI

Addition of Increasing Amounts of $S_f 6$ to Anti-S_f 6 Rabbit Euglobulin

$S_f 6$ N added	Total N ppt.	1.0 ml. antiserum fraction per tube		
		Antibody N by difference	Ratio antibody N: lipoprotein N in ppt.	Tests on supernatant
mg.	*mg.*	*mg.*		
0.0031	0.0336	0.0305	9.98	Excess antibody
0.0061	0.0532	0.0471	7.69	" "
0.0092	0.0617	0.0525	5.72	" "
0.0122	0.0729	0.0607	4.86	No antibody, no antigen
0.0153	0.0841	0.0688	4.50	" " " "
0.0186	0.0954	0.0770	4.20	" " " "
0.0214	0.1036	0.0822	3.84	" " " "
0.0245	0.1149	0.0904	3.69	" " " "
0.0306	0.126	0.0954	3.12	" " " "
0.0459	0.0756			Excess antigen
0.0612	0.0392			" "
0.0765	0.0224			" "
0.0918	0.0112			" "
0.1071	0.0112			" "
0.1836	0.0056			" "

TABLE VII

Addition of Increasing Amounts of S_f 13 to Anti-S_f 6 Rabbit Euglobulin

$S_f 13$ N added	Total N ppt.	1.0 ml. antiserum fraction per tube		
		Antibody N by difference	Ratio antibody N: lipoprotein N in ppt.	Tests on supernatant
mg.	*mg.*	*mg.*		
0.0073	0.028	0.0208	2.86	Excess antibody
0.0109	0.0385	0.0241	2.22	" "
0.0145	0.049	0.0345	2.38	" "
0.0182	0.056	0.0379	2.09	No antibody, no antigen
0.0236	0.0735	0.0499	2.11	" " " "
0.029	0.0875	0.0686	2.01	" " " "
0.0363	0.105	0.0688	1.90	" " " "
0.0453	0.119	0.0737	1.63	" " " "
0.0544	0.0945			" " " "
0.0725	0.0525			Excess antigen
0.0906	0.028			" "
0.1088	0.021			" "
0.145	0.014			" "

TABLE VIII

Addition of Increasing Amounts of S_f 6 to Anti-S_f 13 Rabbit Euglobulin

S_f6 N added	Total N ppt.	1.0 ml. antiserum fraction per tube		
		Antibody N by difference	Ratio antibody N: Lipoprotein N in ppt.	Tests on supernatant
mg.	*mg.*	*mg.*		
0.0022	0.0020			Excess antibody
0.0044	0.0086	0.0042	0.954	" "
0.0066	0.022	0.0154	2.35	No antibody, no antigen
0.0088	0.0321	0.0233	2.67	" " " "
0.0109	0.0366	0.0257	2.35	" " " "
0.01313	0.0441	0.0310	2.36	" " " "
0.0153	0.050	0.0347	2.27	" " " "
0.0175	0.058	0.0405	2.31	" " " "
0.0197	0.040			" " " "
0.0219	0.028			Excess antigen
0.0328	0.014			" "
0.0438	0.0080			" "
0.0547	0.0020			" "

TABLE IX

Addition of Increasing Amounts of S_f 13 to Anti-S_f 13 Rabbit Euglobulin

S_f13 N added	Total N ppt.	1.0 ml. antiserum fraction per tube		
		Antibody N by difference	Ratio antibody N: lipoprotein N in ppt.	Tests on supernatant
mg.	*mg.*	*mg.*		
0.0029	0.0084	0.0055	1.90	Excess antibody
0.0058	0.0196	0.0138	2.34	" "
0.0073	0.028	0.0208	2.86	No antibody, no antigen
0.0087	0.0336	0.0249	2.86	" " " "
0.0102	0.0392	0.0290	2.86	" " " "
0.0116	0.0448	0.0332	2.86	" " " "
0.0145	0.0504	0.0359	2.48	" " " "
0.0159	0.056	0.0401	2.52	" " " "
0.0174	0.0672	0.0498	2.86	" " " "
0.0203	0.0728	0.0525	2.59	" " " "
0.0232	0.0784			Excess antigen
0.0261	0.0672			" "
0.029	0.0448			" "
0.0435	0.0112			" "
0.058	0.0112			" "

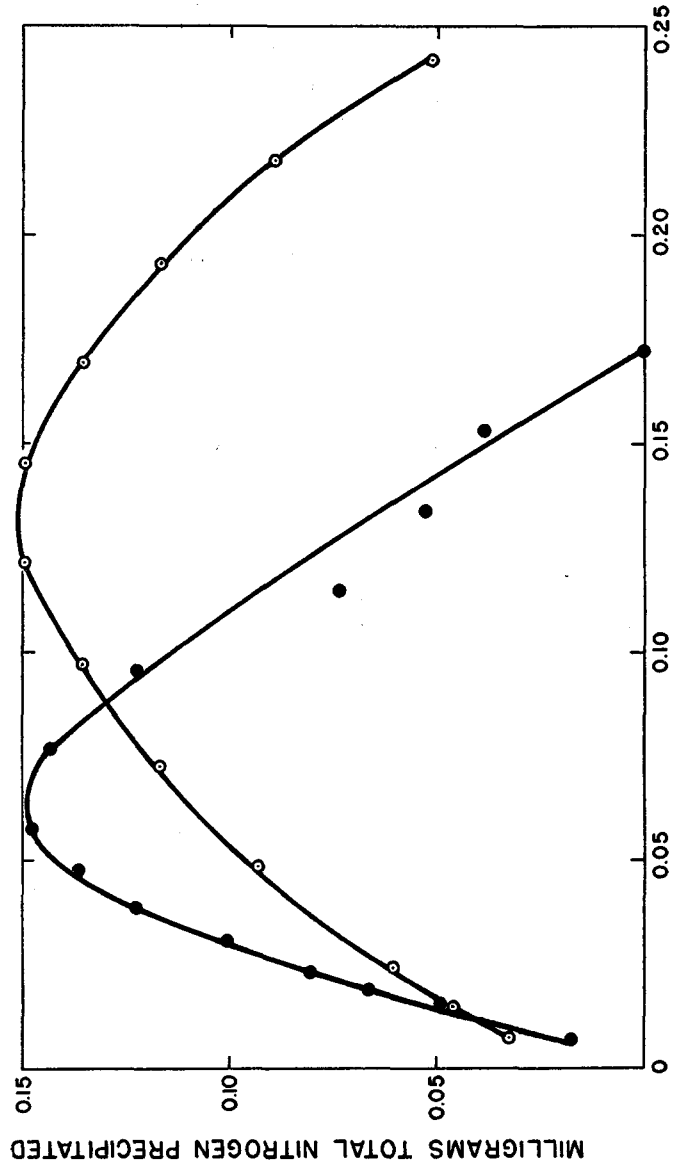

FIG. 2. Addition of increasing amounts of ●●● *Sy6* to rabbit anti-*Sy6* pseudoglobulin, Table II and OOO *Sy13* to rabbit anti-*Sy6* pseudoglobulin, Table III.

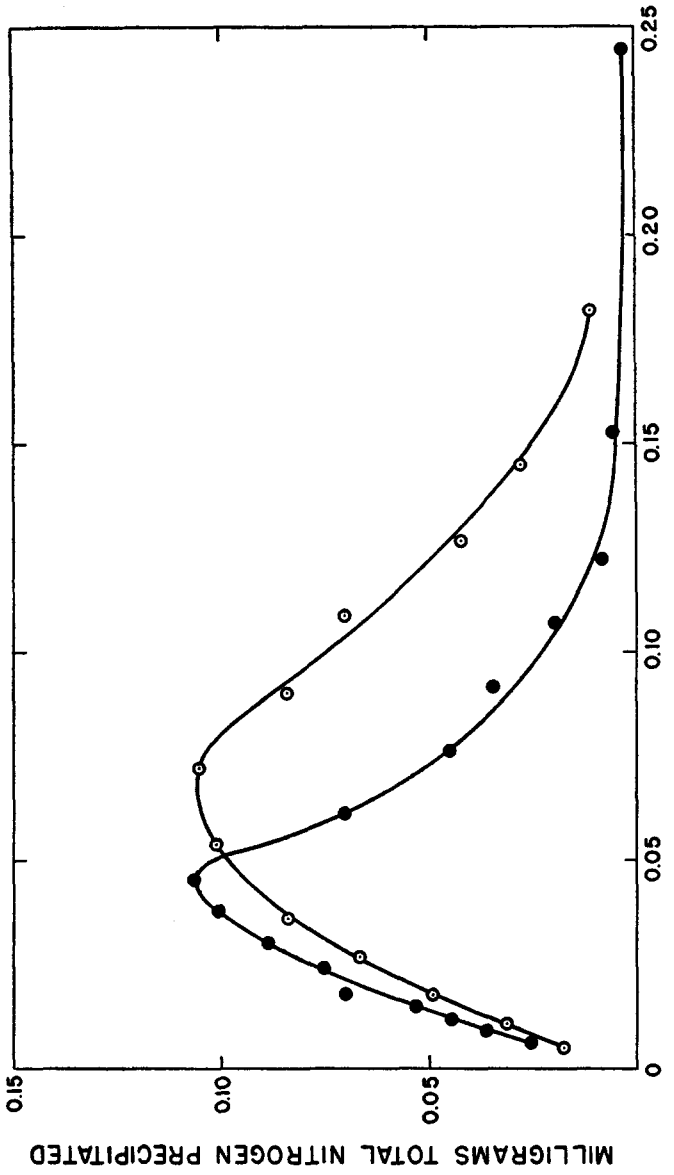

FIG. 3. Addition of increasing amounts of ●●● *Sy6* to anti-*Sy13* rabbit pseudoglobulin, Table IV and ○○○ *Sy13* to anti-*Sy13* rabbit pseudoglobulin, Table V.

days. Antibody preparations P-6, E-6, P-13, E-13 from the second bleeding, and anti-high density lipoprotein serum, each in several concentrations, were reacted with the following antigens: whole serum, isolated S_f17+, S_f13, S_f6, preparations of high density lipoproteins and a lipoprotein-free serum protein preparation; the latter was used as antigen control.

All tubes containing anti-low density lipoprotein serum preparations gave essentially similar results; 6 to 8 bands were observed. However, these bands did not develop in succession, at the leading edge of precipitation. Rather, a precipitation zone formed first. The boundary of this zone moved. After 5 days,

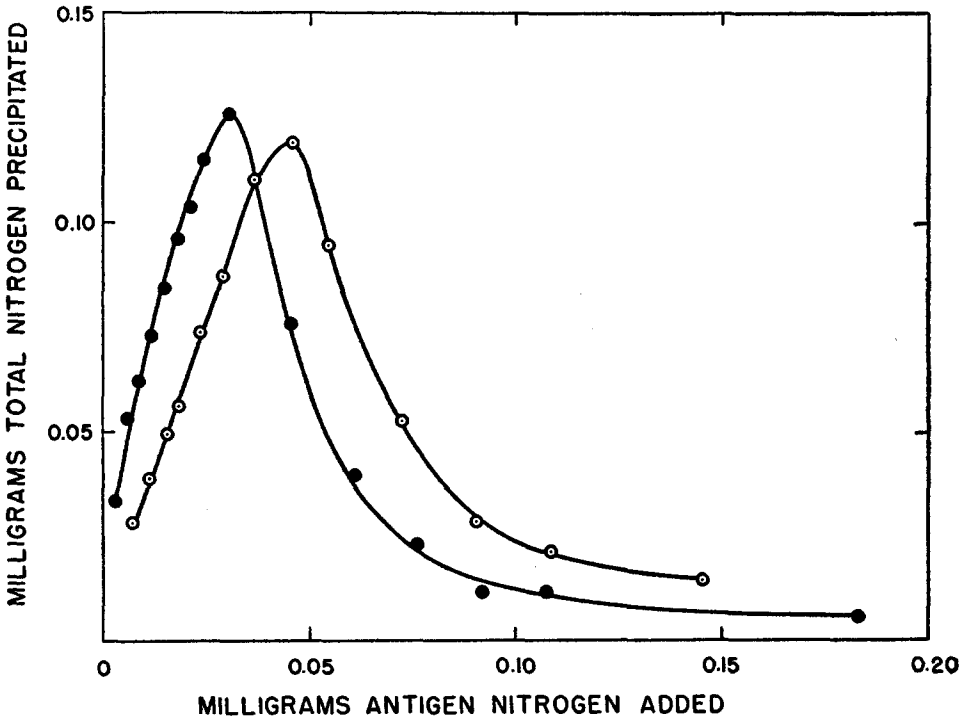

FIG. 4. Addition of increasing amounts of ●●● S_f6 to anti-S_f6 rabbit euglobulin, Table VI and ○○○ S_f13 to anti-S_f6 rabbit euglobulin, Table VII.

within this zone of precipitation, one band was clearly distinguishable and after 7 days 6 bands could be recognized, all within the zone of precipitation. Toward the end of the observation period there were usually 8 bands. Details, like the time of appearance of the bands and the rates of migration were, of course, dependent upon the particular conditions of the experiment, primarily antigen-antibody ratio and concentration, and the temperature of incubation.

Fig. 6 indicates the migration of the leading boundary of 2 concentrations of E-6, and whole serum as antigen. This result is representative for the low density lipoprotein antigen-antibody system.

When agar diffusion tubes were centrifuged at 1000 R.P.M. for a few minutes, some of the precipitates which appeared as bands floated to the top of the tube, while others sedimented.

The migration of bands upwards, into the antigen layer, was observed at certain antigen-antibody ratios, or when sufficient amounts of S_f 17 and higher S_f lipoproteins were present.

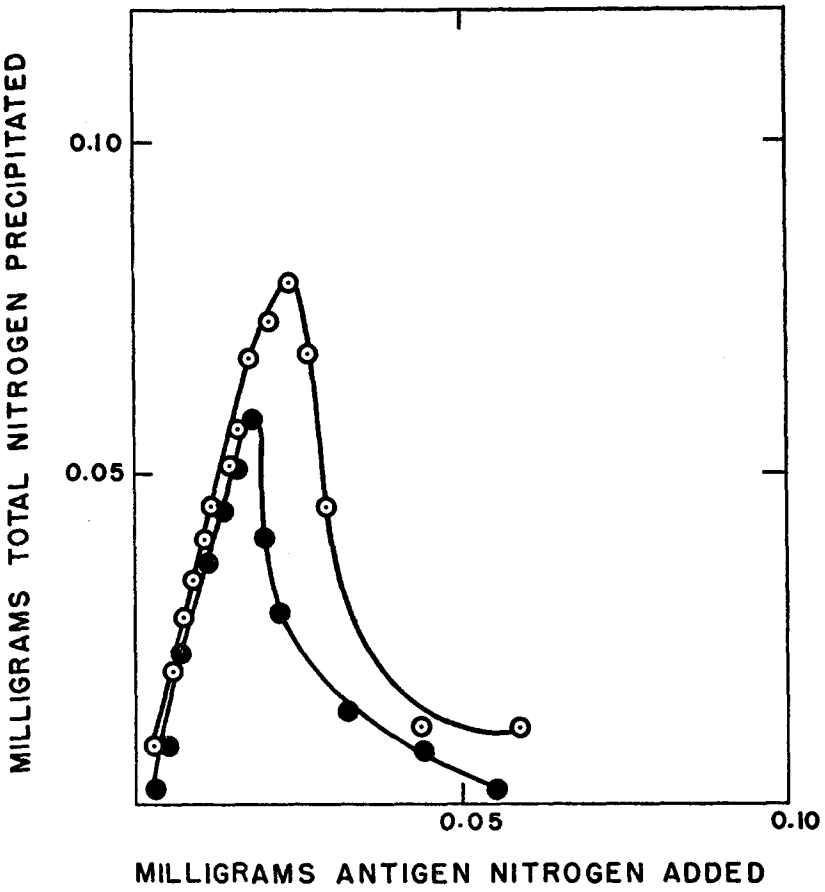

FIG. 5. Addition of increasing amounts of ●●● S_f6 to anti-S_f13 rabbit euglobulin, Table VIII and ○○○ S_f13 to anti-S_f13 rabbit euglobulin, Table IX.

With S_f 17+, 13 and 6 lipoproteins and anti-high density lipoprotein serum, a precipitation zone was observed and within this zone bands appeared. The zone and bands were of the same type as those observed with the anti-low density lipoprotein serum fractions. However, when isolated high density lipoprotein fractions or whole serum were used as antigen, the results were different. For about 10 days there was only one zone of precipitation. Then one

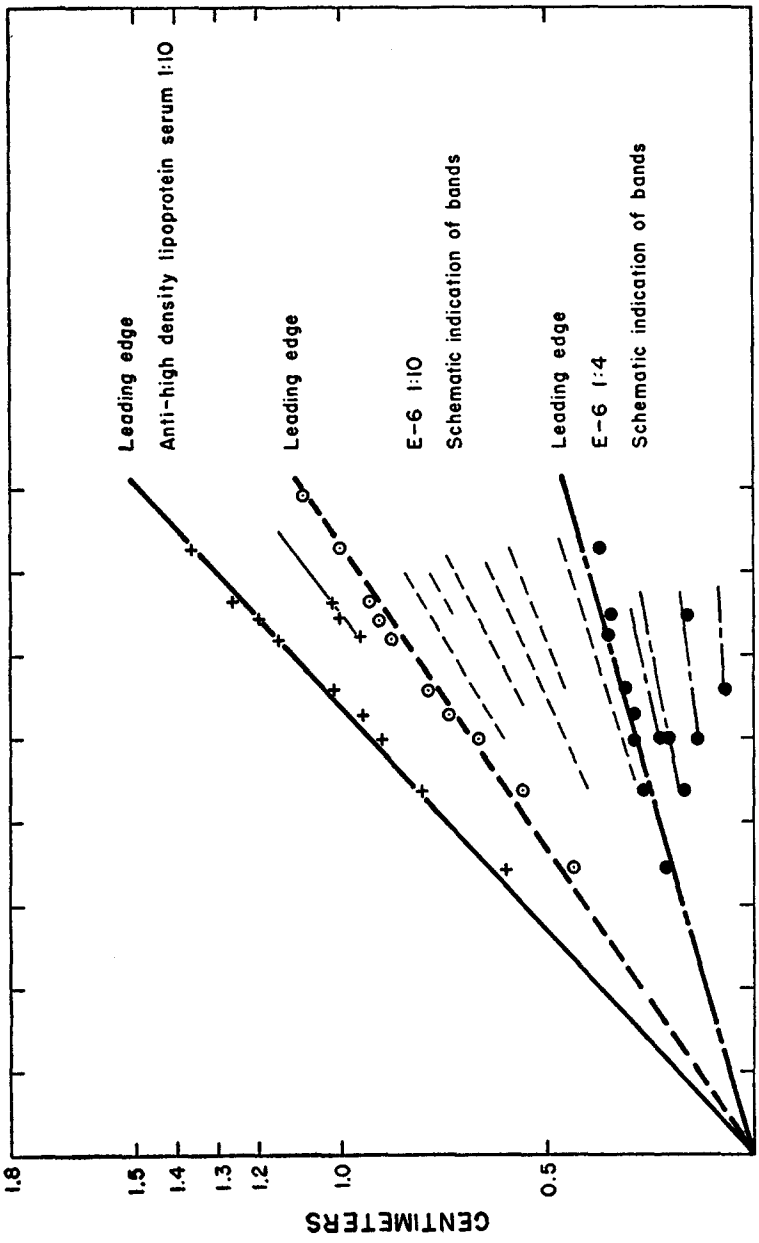

FIG. 6. Agar diffusion analysis. Antigen: 0.5 ml. whole human serum. Antibody: as indicated, 2 ml. Total agar concentration: 0.3 per cent. Temperature 37°C.

new zone became recognizable, as indicated in Fig. 6. This zone also formed within the zone of precipitation but was broad and very different in appearance from the bands that were observed with the isolated low density lipoprotein antigens. No band formation analogous to what was described above was observed here. Interpretations of these findings will be discussed below.

DISCUSSION

The immunochemical homogeneity of a number of different lipoprotein preparations has been examined by absorption, precipitin, and agar diffusion techniques.

Qualitative absorption tests showed that either of the low-density lipoproteins, S_f 6 or S_f 13, can absorb all the precipitating antibodies of both the homologous and heterologous antiserums and antiserum fractions. The data summarized in Table I suggest that lipoproteins have the following minimal antigenic compositions: Low density lipoproteins S_f 6 and S_f 13 contain a set of antigens different than HDL -2 and HDL -3. High density lipoproteins HDL -2 contain at least two antigenic components, one of which is also present in low density lipoproteins. Neither component is present in HDL -3. High density lipoproteins HDL -3 contain at least one antigenic component which is not present in any other lipoprotein.

Quantitative precipitin analyses confirmed the close immunochemical similarity of S_f 6 and S_f 13 lipoproteins. Three criteria of immunochemical homogeneity (11) have been met for these systems: Lipoproteins did not cross-react with non-lipide–containing plasma proteins; in no instance were both antigen and antibody present in the same supernatant; antigen added was completely precipitated throughout the region of antibody excess. Cholesterol analyses of precipitates and supernatants confirmed these findings.

In precipitin systems containing the pseudoglobulin antiserum fractions P-6 or P-13, the amount of lipoprotein nitrogen necessary to precipitate the maximum amount of antibody nitrogen was essentially the same, whether S_f 6 or S_f 13 were used. To reach the point of maximum precipitation with S_f 6 required only slightly more lipoprotein nitrogen than was necessary to precipitate the maximum amount of antibody. With S_f 13, however, maximum amount of precipitation required two or three times the quantity of antigen that sufficed for precipitation of maximal amounts of antibody nitrogen. This was found to be the case with the homologous as well as the heterologous antiserum fractions. In systems containing euglobulin antiserum fractions E-6 or E-13, the antigen concentrations at which the maximum amount of antibody precipitated coincided with that at which the maximum amount of total precipitate formed. But again, more S_f 13 nitrogen was required than S_f 6 nitrogen. Although it is known that the ratio of S_f 6 nitrogen to S_f 13 nitrogen is about 1.2 (12), the lower nitrogen content of S_f 13 cannot account for the observed

differences in nitrogen precipitated unless one makes arbitrary assumptions about the configurations of the lipoproteins. The antigen-antibody ratios with euglobulin antibody fractions were higher than the corresponding ratios with the pseudoglobulin antibody fractions. A similar observation has been previously reported for the ovalbumin- rabbit antiovalbumin system (7).

Since Kunkel (13) demonstrated antigenicity of human plasma lipoproteins, precipitin analyses with lipoproteins have been reported by Gitlin (14) and, while this manuscript was in preparation, by Levine *et al.* (15). Gitlin studied the precipitin behavior of β-lipoproteins isolated by Oncley's method (16). He found that β-lipoproteins isolated from three different plasma pools precipitated differently. On the basis of this result and the finding of multiple bands by the agar diffusion procedure, he concluded that β-lipoproteins are immunochemically markedly heterogeneous. This conclusion would appear to lead to the further conclusion, that the plasma pools, from which β-lipoproteins were isolated, contained varying amounts of immunochemically different lipoproteins. However, the plasma pools were derived from 100 or more pints of blood (17). In the light of what is presently known about the lipoprotein distribution in the human population (18)—as determined by ultracentrifugal methods—large pools would be expected to be of nearly identical lipoprotein composition. It is suggested that results like those reported by Gitlin could have been obtained with lipoprotein preparations obtained from one pool, *i.e.*, even with identical starting material, had there been slight differences in the method of preparation of the β-lipoproteins. Levine *et al.* (15) reported complement fixation and quantitative precipitin analyses of low density lipoproteins. Their quantitative precipitin analyses confirmed our previous report (19) that either of the low density lipoprotein antigens, S_f 6 and S_f 13, can absorb all the precipitating antibodies from the homologous and heterologous antiserums or antiserum fractions, that there are considerable differences in the precipitin curves when different lipoprotein antigens are used with any one antiserum, and that analyses of precipitates for cholesterol indicated that all the lipoprotein antigen was precipitated throughout the region of antibody excess. Levine *et al.* concluded that their antiserum did not differentiate between four low density lipoprotein preparations. Actually, their quantitative precipitin data do appear to show differences between the heterologous and homologous systems, both in quantity of antibody precipitated, as well as the amount of antigen necessary to reach equivalence and antigen excess. These differences were even greater than the ones we have found; our antigen/antibody ratios were of the same order of magnitude as those reported by Levine *et al.*

The interpretation of the agar diffusion data presents some difficulty. Using the Oudin technique, Gitlin (14) observed 6 to 8 bands. Korngold and Lipari (20), employing the Ouchterlony modification of the agar diffusion technique (21), found only one band between antiserum prepared against lyophilized

fraction III (Cohn's procedure 6) and antigen obtained by "centrifuging fraction I + II + III at density less than 1.063." They suggested that material other than lipoprotein may have been responsible for the multiple bands observed by Gitlin. Levine *et al.* also used the Oudin procedure and observed several zones of precipitation between low density lipoprotein preparations and anti-low density lipoprotein serum. They reported that some of the bands were caused by other than lipoprotein systems. After absorbing their antiserum with a lipoprotein-free plasma solution, they found only one band between a low density lipoprotein preparation and the absorbed antiserum. This was interpreted to indicate that only one antigen-antibody system was present. We have two reservations concerning this interpretation: (*a*) their method of preparation of the lipoprotein-free plasma solution probably failed to provide for complete removal of lipoproteins, and (*b*) the time of observation of diffusion in the agar tubes might have been too short for the development of multiple bands. Lipoprotein-free plasma was prepared by centrifuging plasma at density 1.063 gm./ml. for 16 hours at 100,000 *g*; the floated lipoproteins were removed and the subnatant recentrifuged at density 1.21 gm./ml. for 30 hours at 100,000 *g*. The subnatant was assumed to be lipoprotein-free plasma. In the present study we have found that at least two recentrifugations of the subnatant at density 1.21 gm./ml., each followed by removal of the floated lipoproteins, were necessary to remove all the lipoproteins which react with anti-low density lipoprotein serum. Failure to remove the small amount of high density lipoproteins that would presumably be present in the preparation would lead, when absorption with this material is carried out, to absorption of all those antibodies which cross-react with high density lipoproteins. As to the diffusion time, the reported time of Levine and his associates of observation of the agar tubes was only 4 days. It can be seen from Fig. 6 that band formation in the present study was not observed until after the 5th day.

Since our antiserums did not precipitate with human serum albumin, human serum gamma globulin or the lipoprotein-free protein fraction prepared from the subnatant of a 1.2 density run, it is concluded that the bands observed in our agar diffusion analyses were due to lipoprotein-antilipoprotein precipitations. Neither Gitlin nor Levine *et al.* reported the peculiar type of band formation that we observed nor could we find any other report in the literature. Owen (22), applying the double diffusion technique (23) to a whole human serum–rabbit antiserum system, observed a similar phenomenon,—formation of a precipitation zone with subsequent development and migration of the bands within the zone. In both, his experiments and ours, temperature variations were excluded as a possible source of error.

The conclusion that lipoproteins move as a single component in an agar diffusion system (15, 20) must be reexamined. Although our work on agar diffusion does not permit a final conclusion, we are inclined to believe at this

time that each band really represents a distinct though closely related antigen-antibody system.

On this assumption, our results suggest the following antigenic composition of lipoproteins: lipoproteins of highest hydrated density, HDL-3, are antigenically distinct from all other lipoproteins. HDL-2 contain several antigenic components, at least one peculiar to HDL-2, others in common with low density lipoproteins. Continuing the hydrated density scale of lipoproteins towards lower densities, new antigenic components become apparent. Low density lipoproteins S_f 6 and S_f 13 contain at least 6 or 8 components. It might be expected that in lipoproteins of still lower hydrated density additional antigenic components may be present, but that some of those associated with S_f 6 or S_f 13 might be absent. This type of distribution might be thought to exist for lipoproteins up to the chylomicron range.

The basic reason that the reported experiments did not permit clearer separation of antigenic components of lipoproteins might have been the use, throughout this work, of antiserums prepared in the rabbit. This animal's antibody-producing mechanism may be unable to distinguish subtle antigenic differences of materials of human origin. The production of anti-human lipoprotein serums in primates (other than man) might yield much more definitive results.

SUMMARY

Low density human plasma lipoproteins S_f 17+, S_f 13, and S_f 6, high density lipoproteins 2 and 3, and a lipoprotein-free plasma protein fraction were isolated from human plasma by ultracentrifugal methods. It was found that human plasma lipoproteins are immunochemically distinct from the non-lipoprotein containing plasma protein fraction.

Lipoprotein fractions of a given hydrated density, isolated from different individuals, were found to be immunochemically indistinguishable by qualitative absorption tests.

Qualitative antigenic differences were shown to exist between low density lipoproteins and high density lipoproteins.

Quantitative precipitin reactions showed that low density lipoproteins S_f 6 and S_f 13 were immunochemically very similar. However, they differed with respect to the amount of antigen nitrogen required for maximum precipitation.

Agar diffusion analyses were performed; the results suggest heterogeneity of lipoproteins by this criterion.

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